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THE EFFECTS OF CONTINUOUS IRRADIATION BY TRITIUM ON CELLS CULTIVATED *IN VITRO*

BY

AGNES N. STROUD

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THE EFFECTS OF CONTINUOUS IRRADIATION BY TRITIUM ON CELLS CULTIVATED *IN VITRO*

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Division of Biological and Medical Research,
Argonne National Laboratory, Lemont, Ill.

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Introduction

Tissue culture of living cells provides convenient material for use in comparing the relative efficiencies of different qualities and time patterns of radiation in causing delay in cell division, as well as in the morphologic study of aberrations associated with mitotic delay or inhibition.

It has been well established by Canti,[5] Spear,[19] Lasnitzki,[12] Cox,[7] and others, that, in tissue cultures, γ rays, X rays, and hard β rays cause a temporary inhibition or cessation of mitosis followed by recovery at some time during or after the end of the radiation period. The effect is dose-dependent. Large doses permanently stop division whereas, with smaller doses, the effect may be temporary and the recovery complete.

Most of the published information in this field relates to the effects and recovery following a single radiation dose. Very little is known about the mitotic behavior of the cell during continuous irradiation. The injury and recovery constants postulated by Lea[13] suggest that under threshold conditions the rate of cell division may be more or less constantly reduced throughout the course of irradiation. Since mitotic indices are often taken to indicate rates of cell multiplication, it is of interest to know the degree to which completion of visible mitosis is delayed under continuous irradiation. Previous data from this laboratory[1,2,3,21] indicate that in a radiation flux of suitable intensity there is a prolonged reduction in mitotic rate and also a delay in visible mitosis, primarily in prometaphase or metaphase.

A uniform radiation source for continuous irradiation is provided by the use of tritium oxide ($H^3{}_2O$) in the medium. Cultures may be irradiated conveniently and safely while under microscopic observation, since the extremely low energy of the β ray is shielded completely by a glass coverslip. The present experiments were designed to investigate the mitotic behavior of the cell and the nature of mitotic injury during such continuous irradiation. Observations were made on Feulgen-stained cultures and on time-lapse cinematographs and serial photographs of living cells in tissue cultures.

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*The work described in this paper was performed under the auspices of the United States Atomic Energy Commission, Washington, D.C.

Materials and Methods

Fragments of 9- to 10-day chick-embryo limb muscle were cultured by the hanging-drop method or by the microtissue-slide technique for phase microscopy of living cells. The medium consisted of cockerel plasma and 40 per cent chick embryo extract. The diluent for the extract was Tyrode's solution containing tritium oxide. The hanging-drop preparations were incubated for 24 hours at 37° C., then examined under the microscope and placed in groups of approximately equal mitotic rates and growth radii before adding tritium. After treatment, selected cultures were washed in Tyrode's solution, fixed in Bouin's solution, and stained by the Feulgen method. In most cases separate control cultures were used for each radiation group.

In the Feulgen-stained preparations of both control and treated cultures, cells in mitosis and resting cells (normal and abnormal) were counted and the stages of mitosis were classified. Our classification was as follows: *prophase,* from the time when the chromosomes are first visible (spireme) up to the time when the nuclear membrane breaks down and the chromosomes begin lining up at the equatorial plate; *metaphase,* the period during which the chromosomes are lined up parallel at the equatorial plate; *anaphase,* from the beginning of separation of the chromosomes through completion of the cleavage furrow; and *telophase,* up to the point of complete separation into two cells. The ring-shaped areas selected for making counts were at the zone of densest migration (zone III of Mayer[16]. The counting area was a 1.8-mm.^{2} section representative of the respective zone. In each preparation at least 1000 cells were observed. In making counts of cells in the different mitotic stages the "abnormal" mitoses were enumerated separately. These included obvious chromosome breaks, bridges, and various other abnormal states seen primarily in metaphase and anaphase. According to the criterion used, about 10 per cent of the mitoses in unirradiated cultures were observed to be abnormal. Degenerated and exploded cells were also counted.

When radiation effects on living cells were to be observed, 24-hour tissue cultures were transferred to phase-microtissue slides to fresh medium containing tritium. For observation of immediate and delayed effects a treated culture was placed under a phase microscope (Cooke, Troughton, and Simm's) that was contained in a lucite incubator at a constant temperature of 37°C. A 95 X phase (N.A., 1.3) oil objective was used. Motion pictures were taken with a Cine-Kodak Special 16 mm. camera operating at 6 frames per minute. By this method, the length of time during which a cell remained in normal mitosis, or was blocked in any phase of mitosis, was easily determined.

Although the cinematographic system was satisfactory for studying individual mitoses in great detail, it was found necessary to photograph larger fields at frequent intervals in order to determine the total duration of mitosis and the rate at which cells were entering mitosis. For this purpose, a Bausch and Lomb photomicrographic camera was attached to the phase microscope, and serial photographs were taken at 10-minute intervals on the photographic M plates. From the negatives, which included fields of over 100 cells, resting and mitotic cells were counted, and individual figures were followed through the time series.

It was necessary to employ these three techniques in order to gain a complete picture of the phenomena under investigation: (1) stained cultures gave excellent detail and permitted ready estimation of the relative proportions of the different phases, but told little about the rate of cell division or the duration of delay; (2) cinematographic records limited to chosen cells gave reliable information concerning the events associated with mitotic delay and could be used to measure the total duration of mitosis; (3) serial photographs of large areas of cultures gave direct information concerning the rate of multiplication and the duration of morphological changes. The observations accordingly represent 3 individual experiments using the respective techniques.

Dose rates and total doses in the medium, calculated from the concentration of tritium in the culture, are shown in TABLE 1. In general, the dose rates selected were those bracketing the dose (about 60 rep./hour), which has been found to reduce the mitotic index in regenerating liver by about 50 per cent (Brues and Rietz[3]).* The dose rate to structures within the cell may be expected to be of the same order, on the limited assumption that there is a higher rate of exchange of tritium with the hydrogen of organic cell-constituents, that is, the mean dose to the cell should increase proportionately as its ratio of dry to wet weight increases. The dose rate within the cell may approach or even exceed that in the medium, depending on the extent to which tritium is incorporated into organic compounds by preferential rather than simple exchange. It is probable that this dose rate for most parts of the cell will be slightly less than that in the medium, but in view of the uncertainty all dose rates are expressed in terms of those in the medium employed.

Results

Stained Preparation. TABLE 2, group *A*, summarizes data obtained in the experiment on stained preparations at the two higher dose rates,

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*In the paper by Brues and Rietz[3] this dose rate was erroneously stated at 1 rep/hour, but actually was 1 rep/minute. The expression "rep," commonly used in connection with work on radiation effects of isotopes, means "equivalent (energy absorption) to one roentgen."

TABLE 1

TRITIUM CONCENTRATION, DOSE RATE, AND TOTAL DOSAGES EMPLOYED

Mode of observation	*mc./ml.*	*rep./hour**	*Total accm. rep.*
(1) Periodic cell counts (stained)	12.7	166	222,500,920 & 1300
	31.8	416	222,500 & 1300
(2) Periodic field counts (stained)	1.6	21	1021
	3.2	42	2042
	4.8	62	1970
(3) Cinematographic (continuous)	1.6	21	1021
	230.0	3000	60,000
(4) Serial photographs, living cells	3.6	47	47
	7.2	94	94

*The formula used for calculation is $55EC/_{24}$, where 55 is a constant, 24 represents the hours of the day, E is the mean β energy (assumed to be 5.7 kev), and C is the concentration in mc./ml.

166 and 416 rep./hour. Although each set of cultures has its own controls, data are tabulated only for the combined controls of all seven sets. All phases or stages of mitosis are expressed as per cent of those in each of the individual controls. The information in this table may be looked at from the standpoint either of equal time intervals or of equal accumulated doses. In the former case, we see that total mitosis drops off very sharply at the higher dosage rate after the first interval, while it holds up much longer at the lower intensity of irradiation. Considered on the basis of accumulated dosages, the mitotic index remains constant through 222 r at both intensities, while the proportion of abnormal mitoses increases to about 60 per cent or more. From 500 to 1300 r the cultures at the higher dose rate show a much decreased proportion of cells in all phases of mitosis, while at the lower dose rate there is first an increase (statistically significant) followed by a moderate decrease. The increase may be explained on the basis that the lower dosage rate is sufficient to block cells in prometaphase or metaphase, causing a prolonged duration, whereas the higher dose rate (416 rep./hour), while blocking cells in metaphase, also inhibits entrance of cells into mitosis and causes cell death. The proportion of abnormal mitoses increases gradually with time. The fact that abnormal mitoses do not increase more strikingly with time or with dose rate may be explained by the larger proportion of these cells, which are in a degenerated or exploded state (TABLE 4). The absolute number of normal mitoses falls to about 20 per cent of the controls at the lower dose rate and to less than 5 per cent at the higher rate.

Group *B* of TABLE 2 shows the results of treatment at lower dose rates for longer periods. Since the mitotic counts were done by fields, true mitotic indices were not obtained, although fields were chosen with approximate equal densities of cells. We see here similarly high pro-

TABLE 2

EFFECTS OF CONTINUOUS IRRADIATION ON MITOTIC INDEX (STAINED CULTURES)

Group A. Cells Counted

Dose rate rep./hour	Time hours	Total dose rep.	Cells observed	Cells in mitosis	Cells in abnormal mitosis	Per cent of cells in mitosis			Per cent mitosis abnormal	Total mitosis, per cent of	
						Total	Normal	Abnormal		All controls	Individual controls
166	1.35	222	3136	51	30	1.63 ±.23*	0.67	0.96	58.8	90	131
	3.00	500	3084	69	40	2.24 ±.25	0.94	1.30	58.0	123	138
	5.55	920	4092	41	31	1.00 ±.15	0.24	0.76	75.6	55	49
	7.80	1300	3021	47	36	1.56 ±.22	0.36	1.20	76.6	86	73
416	0.53	222	3153	58	39	1.84 ±.23	0.60	1.24	67.3	101	113
	1.20	500	3064	17	11	0.55 ±.13	0.19	0.36	64.7	30	34
	3.12	1300	3058	14	12	0.46 ±.13	0.06	0.40	85.7	25	23
All controls	0.53-8.10	0	7680	140	15	1.82 ±.15	1.63	0.19	10.7	(100)	

Group B. Fields Counted

Dose rate rep./hour	Fields counted	Time hours	Total dose rep.	Cells in mitosis	Abnormal mitosis	Per cent mitosis abnormal	Per cent of controls
21	102	49	1021	86	48	55.8 ± 5.3*	56
42	111	49	2042	75	64	85.5 ± 4.1	43
62	202	31.5	1970	203	128	64.5 ± 4.2	63
Controls	196	52-70	0	315	25	7.9 ± 2.7	(100)

*Standard error of the mean.

TABLE 3

EFFECTS OF CONTINUOUS IRRADIATION ON MITOTIC STAGES

A. Cells Counted

			Normal mitosis				Abnormal mitosis					
Dose rate rep./hour	Cells observed	Total dose rep.	Pro-phase	Meta-phase	Ana-phase	Telo-phase	Pro-phase	Meta-phase	Ana-phase	Telo-phase	All prophases % of cells	All metaphases % of cells
166	3136	222	8	1	5	7	2	16	9	3	0.32	0.54
	3084	500	8	3	7	11	6	20	11	3	0.45	0.75
	4092	920	3	1	3	3	3	18	6	4	0.15	0.47
	3021	1300	1	0	6	4	6	17	8	5	0.23	0.56
416	3153	222	4	2	6	7	3	24	7	5	0.22	0.83
	3064	500	0	0	1	5	1	7	1	2	0.03	0.23
	3058	1300	0	0	1	1	2	6	3	1	0.07	0.20
All treated	22,608		24	7	29	38	23	108	45	23		
All controls	7680	0	31	39	32	23	0	7	6	2	0.40	0.60

B. Fields Counted

Dose rate rep./hour	Fields counted	Total dose rep.									(Prophases per field)	(Metaphases per field)
20.8	102	1021	9	11	9	9	7	22	16	3	0.16	0.31
41.6	111	2042	1	3	0	7	7	33	14	10	0.07	0.33
62.5	202	1970	14	18	17	26	18	57	30	23	0.16	0.37
Controls	196	0	65	57	68	66	3	9	10	3	0.34	0.33

TABLE 4

DEGENERATED AND EXPLODED CELLS

A. Cells Counted

Dose rate rep./hour	*Cells observed*	*Total dose rep.*	*Time hours*	*Degenerate cells (%)*	*Per cent excess of degenerate cells**	*Exploded cells (%)*
166.5	3136	222	1.35	9.82	4.47	0.10
	3084	500	3.00	15.66	5.17	0.23
	4092	920	5.55	11.11	7.11	0.14
	3021	1300	7.80	9.43	5.11	0.33
416.5	3153	222	0.53	5.11	0.82	0.59
	3064	500	1.20	8.71	4.42	2.91
	3058	1300	3.12	7.91	6.03	3.20
All controls	7680	0	0.53-8.10	4.65	--	0.06

B. Fields Counted

Dose rate rep./hour	*Fields counted*	*Total dose rep.*	*Degenerate cells per field (%)*	*Per cent excess of degenerate cells*	*Exploded cells per field (%)*
21	102	1021	2.17	0.67	0.47
42	111	2042	2.57	1.07	1.90
62	202	1970	5.66	4.16	4.90
Controls	196	0	1.50	--	0.15

*Excess over the individual controls for each dose

protions of abnormal mitotic figures where a total dose of 1000 to 2000 rep. has been accumulated. It is also noted that mitosis continues to take place for one to two days, probably at a diminished rate, even where the total doses, if they were given all at once, would inhibit all cell division.

The proportions of mitotic figures in all stages are presented in TABLE 3. The most striking feature here is a marked shift from normal metaphases towards abnormal metaphases, which occurs in all of the irradiated groups more or less independently of dose rate or total dose. It will also be seen that, with those dose rates causing a decrease in the mitotic index (that is, 500 and 1300 rep. at 416 rep./hour), the percentage of prophases is much more reduced than that of metaphases. This suggests inhibition of entry into prophase, combined with a delay at metaphase or, alternatively, a more rapid passage through visible prophase. Where the cells have been treated at lower dose rates (TABLE 3, *B*) a larger proportion of normal mitoses is seen than at higher dose rates (TABLE 3, *A*), although abnormal figures are still in excess.

Of the mitotic abnormalities encountered in irradiated cultures at the higher dosage rates, more than half (100 of 198 abnormal mitoses)

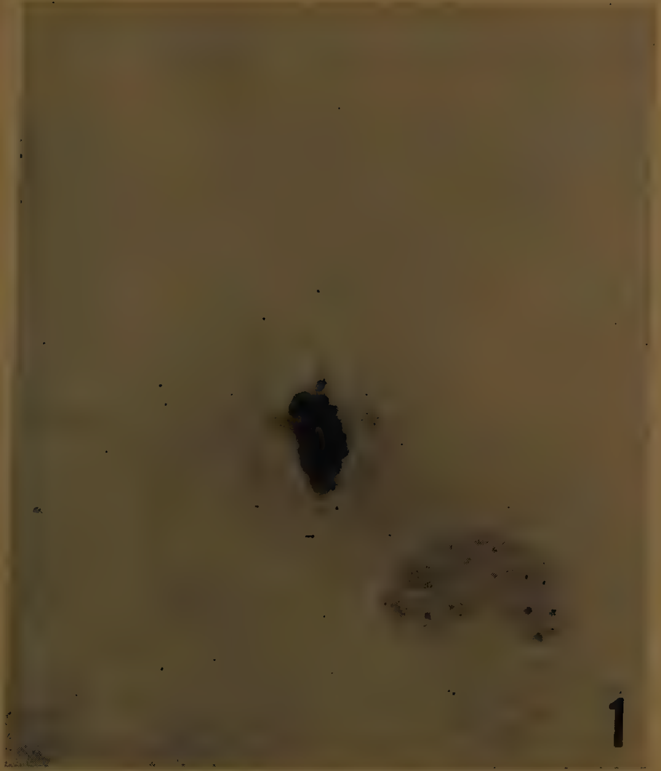

FIGURE 1. Clumped chromosomes at metaphase.

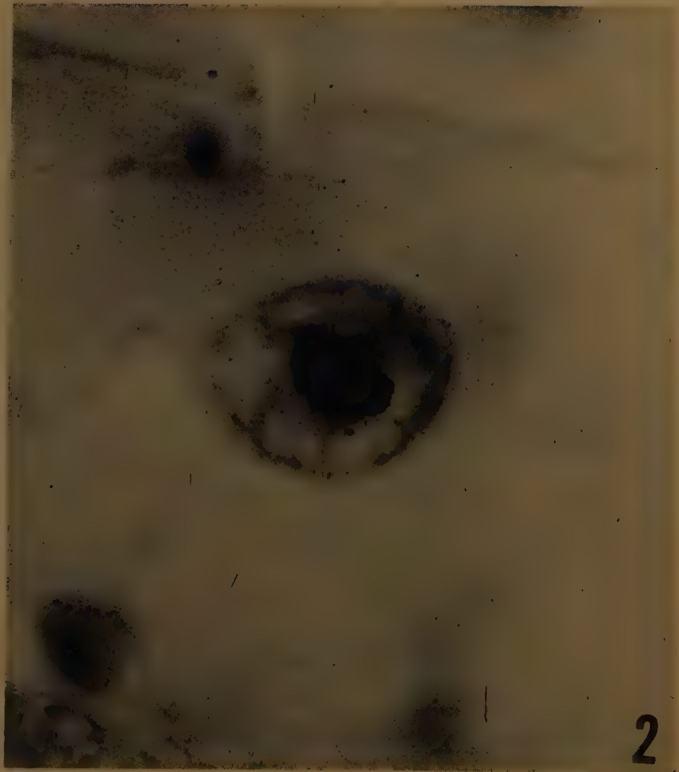

FIGURE 2. An asymmetrical intrachange as the result of a chromosome break.

showed clumping (FIGURE 1), which is assumed to be due to chromosome stickiness. Only 6 breaks, or fragments of chromosomes broken off (FIGURE 2), and 3 bridges (FIGURE 3) were identified in irradiated cells. Twenty-six cells were observed with lagging chromosomes, indicating delay in attachment to the spindle (FIGURE 4). In 24 cells the chromosomes were in a disorganized pattern. Since the nuclear membrane in these cells was broken down, they were not in prophase, therefore the scattered chromosomes appeared to be the result of an incomplete separation at anaphase (FIGURE 5). Ten of the cells in telophase showed depletion of DNA (as seen by the loss of stainable Feulgen material for the chromosomes) and there were 10 in earlier stages of mitosis in which the chromosomes were "stringy" in appearance (FIGURE 6). Two triasters were seen (FIGURE 7). In the remaining 11 mitotic cells the chromosomes were seen as short, sticky, in rosettes or star-clusters (FIGURE 8). The most remarkable finding is the scarcity of structural changes (breaks and bridges) that made up only 2 per cent of control and 3 per cent of all experimental mitotic figures. We are not ruling out the fact that possible structural breaks cannot be identified, particularly when chromosomes become "sticky" and swell.

Degenerate and "exploded" cells generally occur when cells are delayed in prometaphase or metaphase or when, after high intensities of irradiation cells in the resting stage go into division. Various types were seen here, for example, breakdown of the nuclear membrane or fragmentation of the nucleus followed by cell death, or "explosive" dissolution of the cell. This was related both to the dose and to the mitotic activity of the tissue, it was noted in the range between 100 and 10,000 r.[12]

Counts of degenerated and "exploded" cells are shown in TABLE 4. After the first hour, the percentage of degenerated cells reached a value a little more than twice that in control cultures, and the number of such cells was little influenced by dose rate or duration of exposure. This excess over control values was between 4 and 7 per cent of the total number of cells, or on the order of 3 or 4 times the normal mitotic index. Exploded cells, on the other hand, were much more numerous at the higher dose rate. After the first hour they reached as high as 5 times the control value at 166 rep./hour and more than 50 times at 416 rep./hour. After 2 to 3 days, they were found also in considerable numbers at the lower dosage rates. In some instances in which the percentage of degenerate cells was unexpectedly high, the values for individual controls were also high. The difference between each experimental group and its individual control has therefore been calculated and is given in TABLE 4. With the higher dose rate not only is the mitotic index reduced but also metaphase is prolonged. A large proportion of these cells

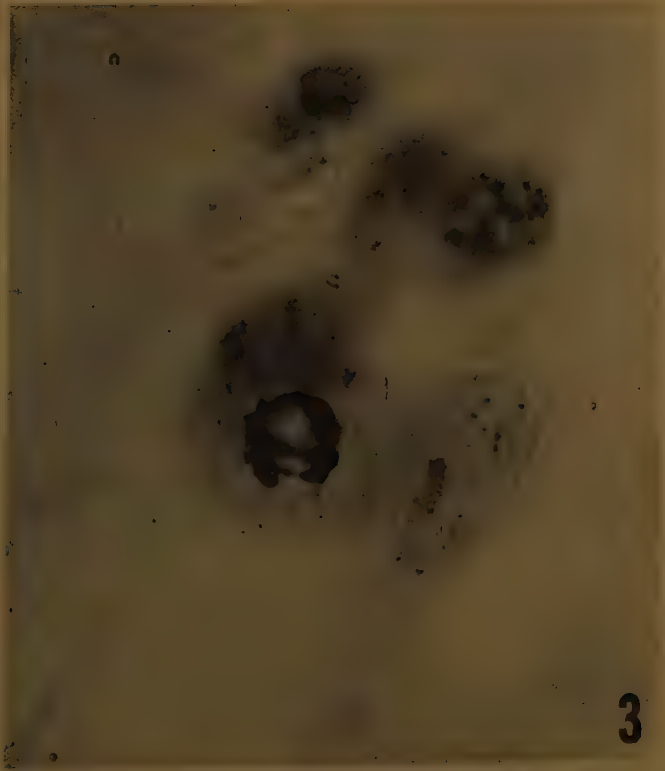

FIGURE 3. Chromosome bridge at anaphase.

FIGURE 4. Lagging chromosome and Feulgen-staining material in interzonal region.

FIGURE 5. Disorganized chromosomes as seen in endomitosis.

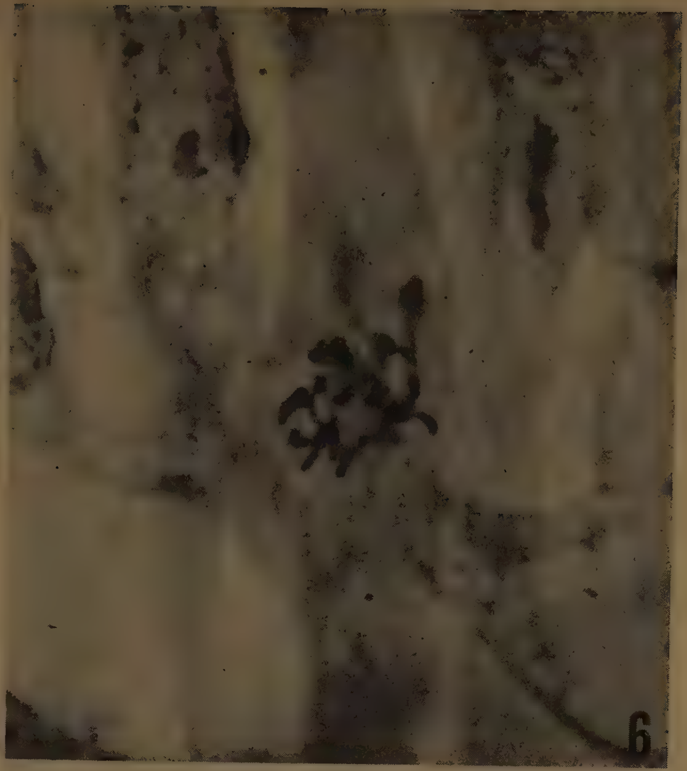

FIGURE 6. Chromosomes with stringy appearance and disorganized at anaphase.

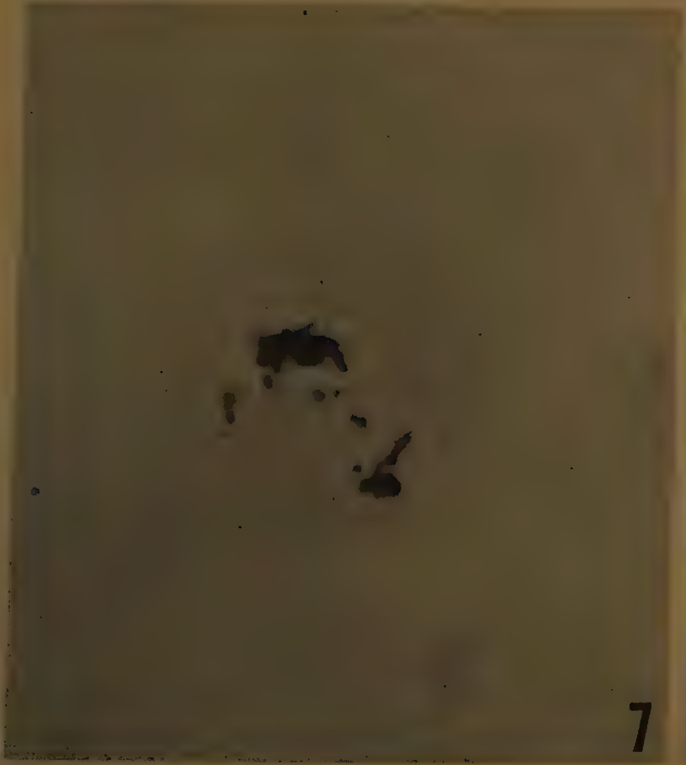

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FIGURE 7. Triaster with markedly unequal distribution of chromosomes.

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FIGURE 8. Rosette or star cluster, showing characteristic arrangement of clumped chromosomes.

will eventually degenerate or explode on division after delay, because of the intensity of the radiation. At the lower dose rate relatively fewer will explode but more will degenerate.

Cinematographic Studies. The cinematographic observation of morphological changes was carried out over a wider range of dosage rates.[21] In the range of 20 to 200 rep./hour most of the mitoses were of longer duration than normal. Since cells were chosen for photography after they had entered prophase, the duration of this stage obviously could not be determined by this technique except as a minimum value, nor could regression in early prophase be determined (TABLE 5). The most striking observations were the extreme variation in duration of mitosis, particularly in metaphase, and the large variety of anomalies in cell behavior associated with the delay. A few figures were seen, even after several hundred rep. had accumulated, that were entirely normal with respect to duration and cell behavior. In TABLE 5, the durations of the various mitotic phases in untreated cells at 37° C. as obtained from our motion picture records, is compared with a number of reports of other workers.[11, 15, 17, 20, 22] Although considerable variation exists between the observations, it will be seen that our own cinematographic observations fall within the limits established by earlier reports. The nature of the abnormalities seen in treated cultures is such as to make definition of the chromosomes in mitosis difficult, particularly if they are delayed in metaphase. In fact, definition was considerably more difficult than in the stained preparations. It is also noticeable that the proportions of mitoses in prophase and telophase are less in stained controls than in the cinematographic records, indicating that these phases are more readily observed in the living cell.

TABLE 5

DURATION OF MITOTIC PHASES OF NORMAL EMBRYONIC CELLS CULTIVATED IN TISSUE CULTURE AT 37° C.

Authors	*Prophase minutes*	*Metaphase minutes*	*Anaphase minutes*	*Telophase minutes*	*Total duration minutes*
Stroud and Brues* (1955)	22	5-13	4-8	14+	45-57
Lewis and Lewis** (1917)	30-60	2-10	2-3	3-12	37-85
Strangeways*** (1924)	15	5	10	15	45
Wright* (1925)	14½	5	4	10	33½
v. Mollendorff+ (1938)	16-24	4-20	4	9-12	33-50
Hughes and Fell‡ (1949)	--	4	2.3-6.0	11-20	17.3-30.3+

*Chick heart muscle.
**Chick mesenchyme.
***Chick choroid.
+Rabbit subcutaneous tissue.
‡Chick frontal bone (osteoblasts).

An outstanding characteristic of the delayed mitosis is the rotation of the group of chromosomes around its central axis, or its shifting in position along the long axis of the cell (perpendicular to the metaphase plate). In such cases the chromosomes usually maintained their relationship to one another, appearing in a transparent gel but lacking, partly or completely, their normal orientation within the cell. It was noted also that metaphase and anaphase sometimes occurred in a gel or mass of increased viscosity that appeared to lack attachment within the cell. In some cases this condition continued for 1 to 2½ hours or longer. In other cases the chromosomes did not remain in a single group but were observed in more or less disorganized groups, as if some of the chromosomes had no spindle attachment. Eventually division often occurred with unequal chromosome distribution to the daughter cells. At higher dose rates (167 rep./hour), linearity of the chromosome group at the equatorial plate was absent, and clumping or disunion of chromosomes from the spindle fiber was frequently seen. Mitochondria were also seen to clump in clusters around the peripheral ends of the chromosome masses. If the cell was delayed in division the mitochondria and other cellular granules, because of their clumping, were unequally distributed when the cell eventually divided. Many cells that underwent a metaphase delay beyond 1½ hours eventually degenerated.

After prolonged exposure at lower dose rates (1500 rep. at 67 rep./hour) there was clumping of the chromosomes and good spindle formation at metaphase but, at the beginning of anaphase marked lagging or temporary bridge formation was seen, which sometimes delayed this phase of mitosis. Of cells in mitosis observed after accumulation of even larger doses (3000 rep.), not only were mitochondria seen around the periphery of the figure, but protoplasmic granules of other sorts appeared in increased numbers in the peripheral cytoplasm. Increased quantities of cytoplasmic material (basophilic in stained preparations) were also seen in resting cells under these conditions of treatment.

Anomalies were also frequently observed in the relation of motion of the cell surface to other phenomena of mitosis. Surface motion is a characteristic accompaniment of the mitotic process occurring normally near the beginning of anaphase and it has been suggested that this reflects a loss of the normal tonicity of the cell surface, resulting from a shift in energy mechanisms in the cell which takes place at a specific time in the mitotic cycle.[14] In cells under irradiation, this surface motion frequently began during metaphase before any evidence of anaphase separation or of spindle formation, and it continued throughout the duration of the delayed figure. This suggests that the motion tends to begin at its usual time irrespective of anaphase movement, independent of the other delays and anomalies. If the process became much prolonged, the motion sometimes became very sluggish. With the accumulation of larger radiation doses it was often sluggish or almost absent from the outset, especially in cells with increased cytoplasmic granule formation. In a few instances surface motion was limited to only a part of the cell surface. It is evident that under irradiation, the characteristic changes in tension of the cell surface lose their normal relation to other events occurring within the cell.

Further evidence of this sort is seen in the behavior of the cleavage furrow that also normally follows spindle formation and chromosome separation at metaphase with great regularity. In some of the cells under irradiation, this regularity was lost both as to time and space. The furrow was seen to develop opposite an equatorial plate that failed to divide and, in such cases, division was discontinued after a short or prolonged period of time. In other cases it appeared at a position somewhat displaced from the plate along the long axis of the cell. In a few cells in which the chromosomes were in a gel, the mass shifted from one to the other polar end of the cell, after which division failed to proceed for periods up to one or more hours. When the constriction of the cell leading to the cleavage furrow first appeared, the chromosome gel immediately oriented itself to the central area from its position at one pole, following which the chromosomes separated toward the poles and cleavage was completed. Thus, the formation of the cleavage furrow can begin independent of the spindle. At other times, a portion of the cell containing no chromosomes or a deficient complement pinched off, suggesting that cleavage occurred prematurely in relation to the mitotic process. In one case 2 separate cleavage furrows appeared as if to divide a triaster, but the cell eventually degenerated without dividing. From all this it is clear that those changes within the cytoplasm that are responsible for the surface changes are independent of the spindle mechanism and chromosome movements.

Changes in nondividing or resting cells were seen only after higher radiation doses. The accumulation in the cytoplasm of granules, shown in stained preparations to be deeply basophilic, has already been mentioned. At accumulated doses above 15,000 rep., exploding cells were seen. The cell surface suddenly expanded to an irregular shape, and this was followed by loss of small granules from the cytoplasm and Brownian motion in those that remained. Almost immediately (within 15 seconds) after surface expansion, the nucleus invariably shrank quickly, leaving a clear area in the space it formerly had occupied.

Another phenomenon seen at high doses was the formation of blisters that were external to the normal cytoplasmic structure. They contained a

TABLE 6

EFFECT OF DELAYED MITOSIS ON MITOTIC INDEX IN IRRADIATED CULTURES

Dose rate rep./hour	*Number of cells followed*	*Cell hours*	*Total mitoses seen*	*Number seen entering*	*Per cent of cells entering mitosis/hour (true rate of mitosis)**	*Mean mitotic index***	*Mean duration in minutes****	*Range of values (minutes)*	*Calculated intermitotic time (hours)+*
0	441	627	10	7	1.11	1.00	40	20- 50	46.2
47	241	482	7	5	1.03	1.37	61	20-120	51.4
94	386	772	14	7	0.93	1.84	75	30-120	47.1

*Number of cells seen entering mitosis of total cells observed.
**Mean of the mitotic indices observed at each time interval.
***Mean of directly observed durations.
+Calculated from mean mitotic indices and mean duration by Hoffman's formula.[9]

few fine particles in Brownian motion and were surrounded by a visible membrane. They varied in size, but were generally not seen to change in size during observation. In some cases they were nearly as large as the cell itself.

Photographs. The observations and measurements given above indicated clearly that visible mitotic phases are prolonged, and they suggested some of the mechanisms but failed to give any notion as to the average duration of delay. They failed also to give a complete picture of the extent of inhibition of the entrance of cells into mitosis during the irradiation of the culture. In the third series of experiments we attempted to obtain such information by taking serial photographs at 10-minute intervals, over 1- to 2-hour periods, of areas of culture containing 75 to 150 cells. The negatives were examined for mitoses, and these mitoses were followed through the successive photographs. This method gives a direct measure of the rate at which cells are entering mitosis and a fair estimate of the mean duration of mitosis (a mitotic figure seen in only two 10-minute photographs was taken to be of the most probable duration of 20 minutes).

TABLE 6 shows the results of this experiment. Since the observations are somewhat laborious (10,669 cells were counted in 100 photographs) the data are necessarily scanty. They indicate that, while the specific rate at which cells enter mitosis (true rate of cell division), decreases slowly with increasing radiation dose, the number of mitoses seen at one time (mitotic index) rises sharply. This is accounted for by the marked increase in the duration of visible metaphases.

The mean mitotic time in these observations differs slightly from those recorded in TABLE 5. From inspection of TABLE 5 it is obvious that the greater part of the mitotic time is in prophase and telophase, in which the criteria used by an observer are uncertain. It seems quite likely that the different conditions of observations account for the differences.

Intermitotic time was calculated by the method of Hoffman[9] for untreated cultures on the basis of data in TABLE 6. Fischer and Parker[8] estimated this period as 62 hours, whereas Olivio and Delorenzi[18] by indirect means calculated it to be between 13 and 21 hours, based on a mitotic time of 12 minutes. Hughes[10] estimates an intermitotic period of 12 hours for tissue cultures of chick or mammalian cells growing under optimal conditions. It is not surprising that cultures grown under different conditions should show considerable variance. The intermitotic time as calculated on our controls is 46.2 hours. In the case of the irradiated cultures the values are almost identical. It should be emphasized that the intermitotic time is calculated in an indirect fashion, and therefore its validity depends on the assumption that a steady state exists, that is, that rate and duration of mitosis and duration of the intermitotic time remain constant. The similarity in intermitotic times of the three groups shown in TABLE 6 reflects the fact, as shown by direct observation, that the rate of entrance of cells into mitosis is not much altered at this time.

Discussion

These observations on cells during continuous irradiation with tritium oxide serve to amplify somewhat our knowledge of radiation effects on intermitotic and mitotic division, which has usually been investigated during recovery from a single irradiation of short duration.

It has been rather generally appreciated that the production of structural chromosome changes (breaks and translocations) is dependent on dose rate. Under the conditions employed here, in which the dose rate is so low that cells continue to divide, and under observations made during irradiation, essentially none of these changes is seen. Another type of change is predominant here, which is seen in stained cultures as "clumping" or a variety of other conditions. Judging from the cinematographic evidence, these changes involve interference with the coordination of the events associated with cell division: gelation phenomena, formation and orientation of the metaphase plate and spindle, initiation of anaphase, surface motion of the cell, and formation of the cleavage furrow. Most striking was the observation that the formation of the furrow is clearly independent of the spindle. In other words, the cytoplasmic conditions responsible for constriction of the surface, whatever they may be, are determined neither by the spindle mechanism nor by chromosome movement.

Another significant observation is that the active surface motion of the cell, normally initiated at the beginning of anaphase, may also occur earlier, in the course of the delayed metaphase. Unpublished cinematographs of cells grown in threshold concentrations of colchicine show certain of the same changes that occur during irradiation, although in colchicine-treated cultures larger proportions of mitoses are merely delayed in metaphase and anaphase without other accompanying abnormalities of behavior.

The mitotic abnormalities seen during irradiation are quite diverse, but apparently normal divisions may occur occasionally at the same time. One is tempted to suggest that these changes are due to "hits" on the spindle fibers, but this seens improbable in view of recent studies of Zirkle and Bloom,[23] who saw no changes in the mitotic process after direct irradiation of the centrosome and spindle with collimated proton beams. It appears rather more likely that the disturbances are related to effects of radiation on the metabolic processes and energy pathways responsible for the highly specialized series of physicochemical events (for example, gelation), that determines the orderly mitotic sequence. That these changes are not prominent after single doses of radiation is due in part to the great predominance of structural chromosome damage where intense dose rates are employed. Quite likely it is due also to the fact that observations are generally made after an interval during which mitosis is either altogether suppressed or has had a chance to recover (thus allowing time for recovery from other effects).

It seems clear from the data presented here that the mitotic index of the fixed culture or tissue does not give a true picture of the rate of cell division. This is a result of the variable duration of mitosis during irradiation, in which the passage of a cell through the visible phases is generally delayed. Because of the variability in duration, the present data do not suffice to indicate what correction factor is applicable to mitotic indices. It may be said, however, that such mitotic indices are certain to give too high a value for the rate of cell division, and that this error is probably greater with an increasing dose rate. One suspects that the prolongation of visible mitosis is less marked in the case of recovery from a single radiation treatment, but this is by no means established. In view of the reliance that has generally been placed on mitotic indices, it would seem important that this be investigated further. In particular, it would be of interest to know to what extent the overcompensation seen at the end of recovery may be an artifact due to cells delayed in metaphase.

A further reflection of this phenomenon is seen in the metaphase: prophase ratio in stained preparations, which rises when the cell is irradiated. This suggests that the prophase index may be a more accurate indicator of the rate of entry of cells into mitosis. The altered ratio might be accounted for by more rapid passage of cells through prophase, but more data are needed to establish this point. It may also be remarked that because of the nature of the abnormalities, it is likely that a given figure might be scored differently on living and fixed material. A late prophase in a rotating gel might appear, for example, after fixation, as a clumped metaphase although, since nuclear membranes were absent here, these were scored as metaphases.

In cells observed under continuous irradiation, inhibition of mitosis, accumulation of metaphases, and cell degeneration are dependent both on dose rate and on total dose. Two phenomena that are clearly more sensitive to dose rate than to total dose (TABLE 3) are depressions of the prophase index and the shift from normal metaphases to abnormal forms.

Various studies on the events following a single irradiation suggest that the present data can be accounted for by theories involving either a dynamic process of injury and recovery or the destruction of a constantly synthesized substance, such as DNA and RNA. Lea[13] has indicated, on the basis of published studies on chick tissue cultures,[6] that mitotic depression after irradiation at various dose rates can be explained simply by assuming a recovery constant of about 200 minutes and a cumulative dose yielding 50 per cent reduction in mitotic activity of about 100 r. Unfortunately, our data fail to show whether or not these constants apply when an equilibrium state is reached during constant irradiation (if such an equilibrium is reached). The implication is that a dose rate on the order of one half r. per minute will result in an equilibrium state in which the rate of cell division is reduced by 50 per cent.[3] Owing to the error introduced by the prolongation of visible metaphases, only the data in TABLE 6 would be valid in such calculations, and these are not extensive enough and do not involve a sufficient irradiation period. It may be noted that experiments with regenerating liver[3] indicate a dose rate of about 1 r/minute for equilibrium effect after 24 to 48 hours. In this case it was impossible to deal with the error in mitotic index caused by prolonged mitosis (nor has it been dealt with in other published studies).

Summary

The effects of continuous irradiation with tritium oxide on chick embryo skeletal muscle tissue cultures have been studied by means of stained preparations, time-lapse cinematographs, and serial photographs of large areas. Irradiation was delivered by the incorporation of tritium oxide into the water of the culture medium, which permitted observations to be made safely during irradiation.

During treatment at low dose rates at the integrated dose levels studied, structural chromosome changes are rarely seen, although a variety of functional abnormalities in the mitotic process occur. These result in a considerable prolongation of visible mitosis, particularly during metaphase. This effect is sufficient to invalidate the use of the mitotic index, under these conditions, as an index of the rate of cell division.

The alterations in the various phenomena associated with mitosis are very diverse, and represent a dissociation of several events that normally are well coordinated. In particular, it is seen that the formation of the cleavage furrow normally occurring at the onset of metaphase and the surface motions of the cell are functionally independent of the state of the spindle and chromosome movement.

The changes that are more closely dependent on dose rate than on total radiation dose are depression of prophase index (which is probably closely related to true rate of cell division) and the proportion of visibly abnormal metaphases.

At high dose rates, degeneration of cells through "explosive" death is a prominent phenomenon. It is invariably accompanied by the accumulation of basophilic material in the cytoplasm..

It is assumed that the changes dealt with in this paper are due to irradiation by the tritium β rays. It is possible that certain of the types of damage observed may be related to the recoil of a tritium incorporated in an organic molecule. This cannot be evaluated because of the lack of comparable observations on cells irradiated continuously with X or γ rays. That no considerable quantitative differences exist may be inferred from the comparable toxicities of X radiation and tritium (equated in physical terms) to whole animals.[4]

While there is no reason to believe that qualitatively different results would be obtained by continuous X or γ-irradiation, there are no data available to establish this. The methods employed do not appear suitable to demonstrate quantitative differences due to differences in linear energy transfer.

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References

1. Brues, A. M. & A. N. Stroud. 1950. Effects of tritium on cells cultivated *in vitro*. Anat. Record. **106**: 181.
2. Brues, A. M., A. N. Stroud & G. Svihla. 1951. Mitotic changes accompanying continuous irradiation. Anat. Record. **109**: 411.
3. Brues, A. M. & L. Rietz. 1951. Effect of external and internal radiation on cell division. Ann. N. Y. Acad. Sci. **51**(8): 1497.
4. Brues, A. M., A. N. Stroud & L. Rietz. 1952. Toxicity of tritium oxide to mice. Proc. Soc. Exptl. Biol. Med. **79**: 174.
5. Canti, R. G. 1927. The effect of irradiation on tissues. London, England. St. Bartholomew's Hosp. Rept. **60**: 57.
6. Canti, R. G. & F. G. Spear. 1927-1928. The effect of gamma irradiation on cell division in tissue culture *in vitro*. Proc. Roy. Soc. B **102**: 92.
7. Cox, S. F. 1931. Tissue culture and its applications to radiological problems—time and intensity factors in dosage. Brit. J. Radiol. **4**: 111.
8. Fischer, A. & R. C. Parker. 1929. The occurrence of mitoses in normal and malignant tissues *in vitro*. Brit. J. Exptl. Pathol. **10**: 312.
9. Hoffman, J. G. 1947. Wright's Hypothesis: Its relation to volume growth of tissue cells and mitotic index. Science. **106**: 343.
10. Hughes, A. 1952. The mitotic cycle. : 1-232. Academic Press, Inc. New York, N.Y.

11. HUGHES, A. F. W. & H. B. FELL. 1949. Studies on abnormal mitosis induced in chick tissue cultures by Mustard Gas (BB'- Dichlordiethyl Sulphide). Quart. J. Microscop. Soc. **90**: 37.
12. LASNITZKI, I. 1943. The response of cells *in vitro* to variations in X-ray dosage. Brit. J. Radiol. **16**: 137.
13. LEA, D. E. 1947. Actions of radiation on living cells. : 1-402. Cambridge Univ. Press. Cambridge, England.
14. LETTRE, H. 1952. Some investigations on cell behavior under various conditions: A Review. Cancer Research. **12**: 847.
15. LEWIS, W. H. & M. R. LEWIS. 1917. The duration of the various phases of mitosis in the mesenchyme cells of tissue cultures. Anat. Record. **13**: 359.
16. MAYER, E. 1939. Tissue cell colonies *in vitro*. Uitgeverij Dr. W. Junk, Den Haag. Tabul. biol. Hague. **65**: 1.
17. v. MOLLENDORFF, W. 1938. Zur Kenntnis der Mitose. Arch. Exptl. Zellforsch. **21**: 1.
18. OLIVIO, O. M. & E. DELORENZI. 1932-1933. Ricerche sulla velocita di accrescimento delle cellule d degli Organi. LLL. Coefficiente mitotico dele'-accrescimento distribuzione topografica e coronologica delle mitosi e durato delle intercinesi nella zona di migrazione delle colture *in vitro* ricavata dall 'osservazione diretta. Arch. Exptl. Zellforsch. **13**: 221.
19. SPEAR, F. G. 1935. Tissue culture II and III. Its application to radiological research. Brit. J. Radiol. **8**: 68.
20. STRANGEWAYS, T. S. P. & H. E. H. OAKLEY. 1923. The immediate changes observed in tissue cells after exposure to soft X rays while growing *in vitro*. Proc. Roy. Soc. B. **95**: 373.
21. STROUD, A. M. & A. M. BRUES. 1954. Radiation effects in tissue culture. Texas Repts. Biol. Med. **12**: 931.
22. WRIGHT, G. P. 1925. The relative duration of the various phases of mitosis in chick fibroblasts cultivated *in vitro*. J. Roy. Microscop. Soc. **45**: 414.
23. ZIRKLE R. E. & W. BLOOM. 1953. Irradiation of parts of individual cells. Science. **117**: 487.

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